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ORIGINAL RESEARCH

Pulmonary adenocarcinoma mutation profile in smokers with smoking-related interstitial fibrosis

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Abstract: Cigarette smoking is an established cause of lung cancer. However, pulmonary fibrosis is also an independent risk factor for the development of lung cancer. Smoking-related interstitial fibrosis (SRIF) has recently been reported. We hypothesized that adenocarcinomas in lungs with SRIF might show distinct molecular changes and examined the molecular phenotype of 168 resected lung adenocarcinomas in lungs with and without SRIF. The diagnosis of SRIF was determined by histological examination, based on the presence of alveolar septal thickening, due to pauci-inflamed, hyalinized, "ropy" collagen, in areas of lung greater than 1 cm away from the tumor. Tumors were concomitantly examined genotypically for mutations in genes frequently altered in cancer, including *EGFR* and *KRAS,* by SNaPshot and by fluorescence in situ hybridization for possible *ALK* rearrangements. Fluorescence in situ hybridization for *ROS1* rearrangement (n=36) and/or *MET* amplification (n=31) were performed when no mutation was identified by either SNaPshot or *ALK* analysis. Sixty-five cases (38.7%) showed SRIF, which was distributed in all lobes of the lungs examined. No differences were observed in sex, average age, or smoking history in patients with and without SRIF. There was no difference in either the percent or types of adenocarcinoma genetic mutations in patients with SRIF versus those without. This data suggests that SRIF does not represent an independent risk factor for the development of the major known and targeted mutations seen in pulmonary adenocarcinoma. However, additional research is required to investigate the potential significance of SRIF in the pathogenesis of lung cancer.

Keywords: lung, cancer, smoking, SRIF

Introduction

Cigarette smoking is the leading cause of lung carcinoma. Lung cancer has also been linked to various fibrosing lung diseases, including idiopathic pulmonary fibrosis (IPF), systemic sclerosis, and dermatomyositis, and certain pneumoconioses (eg, asbestosis and silicosis) such that pulmonary fibrosis may represent an independent risk factor for developing lung cancer.[1–4] However, the role of pulmonary fibrosis in pathogenesis of lung cancer has not been elucidated. Recently, investigators have described a distinct type of pulmonary interstitial fibrosis in smokers, smoking-related interstitial fibrosis (SRIF). SRIF is characterized by the presence of alveolar septal thickening by hyalinized collagen with little associated inflammation and is histologically distinguishable from other fibrosing lung diseases.[5,6] Furthermore, pulmonary fibrosis associated with emphysema has been recognized as a pattern of lung abnormality in the radiographic imaging of smokers and has been associated with an increased risk of lung cancer in prior studies.[7–10] Thus, we hypothesized that SRIF might be associated

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with lung cancer with specific genetic alterations. For this reason, we interrogated multiple genes for patterns of mutations in lung adenocarcinomas in patients with concomitant evidence of SRIF.

Materials and methods

Case selection

The files of the James Homer Wright Pathology Laboratory of the Massachusetts General Hospital were searched for lung specimens resected between 2008 and 2012 with the diagnosis of pulmonary adenocarcinoma. Only cases in which a substantial portion of lung parenchyma uninvolved by tumor was resected (ie, large wedge resections, multiple wedge resections, segmentectomies, lobectomies, or pneumonectomies) were included. Additionally, only cases with tumors on which genetic testing had been performed for clinical purposes were included. Specimens resected from nonsmokers and patients with a prior history of neoadjuvant radiation therapy were excluded. A smoking history was obtained from the patients' medical records and based on self-reporting. If available, imaging studies were reviewed.

Histologic evaluation

Two pathologists (AP and RLK) retrospectively examined hematoxylin and eosin (H&E)-stained sections of lung parenchyma uninvolved by and distant from tumor in order to evaluate for emphysema and SRIF. The number of slides examined was between two and ten per case, depending on the size of the resection specimen and extent of tumor involvement. The criteria used for the diagnosis of SRIF were the presence of paucicellular, eosinophilic, collagenous thickening of alveolar septae in at least 30% of the examined lung parenchyma that was uninvolved by tumor (specifically, >1 cm away from the tumor), with no identified confounding cause. Respiratory bronchiolitis was defined by the presence of pigmented (smoker's) macrophages within the respiratory bronchioles and/or surrounding peribronchiolar alveoli.

Mutational analysis

Tumors (n=168) were examined genotypically for 71 hot spot mutations in 14 genes frequently altered in cancers (including *AKT1, APC, BRAF, B-catenin, EGFR, IDH1, KIT, KRAS, MAP2K1, NOTCH1, NRAS, PIK3CA, PTEN,* and *TP53*) by SNaPshot (Applied Biosystems, Foster City, CA, USA), a previously described mutational analysis assay.[11] Briefly, DNA from formalin-fixed, paraffin-embedded tissue was subjected to a multiplexed polymerase chain reaction (PCR) and single-base extension reaction platform in order to generate fluorescently labeled signals that interrogate hot-spot mutation sites. The SNaPshot products were then analyzed by capillary electrophoresis to determine selected hot-spot mutations. In addition, direct PCR sequencing techniques were used to assay *EGFR* exons 19 and 20 and *ERBB2 (HER2)* exon 20 for in-frame activating insertions or deletions.

Fluorescence in situ hybridization (FISH) was performed on deparaffinized, protease-treated, 5 μm thick sections cut from tumor material from most cases (n=162) in order to evaluate for the presence of *ALK* rearrangement. H&E-stained slides were reviewed to select regions of the tissue that contain mostly tumor cells to be used for hybridization. Dual-color break-apart probes, constructed from BAC clones, specific for the 3′ and 5′ sequences of the *ALK* gene were hybridized according to the manufacturer's protocol using a Hybrite slide processor (Abbott Molecular, Des Plaines, IL, USA). A case was considered positive for *ALK* rearrangement if 15% of cells contained split fluorescent signals.

In tumors in which no mutation was identified by the SNaPshot assay and no *ALK* rearrangement was present, FISH for *ROS1* rearrangement (n=36) and/or FISH for *MET* amplification (n=31) were performed. The presence of *ROS1* rearrangement was evaluated using a protocol identical to that described for *ALK* FISH, with *ROS1*-specific 3′ and 5′ sequence probes. To analyze *MET* gene amplification status, deparaffinized, protease-treated, 5 μm thick sections cut from tumor material (as selected based on review of H&E-stained slides) were hybridized with dual-color probes for the *MET* locus and centromere on chromosome 7 according to the manufacturer's protocol using a Hybrite slide processor (Abbott Molecular). *MET* amplification was considered present when the *MET*: centromere 7 signal ratio was 2.2 or greater.

Statistical analysis

The difference in age and smoking history in patients with and without SRIF was compared by means of an unpaired *t*-test. The Fisher's exact test was used to compare the sex distribution, presence of emphysema, and distribution of examined parenchyma in cases with and without SRIF. The presence of genetic alterations in evaluated genes was compared between lung adenocarcinoma cases with and without background SRIF using the Fisher's exact test. Two-tailed *P*-values less than 0.05 were considered statistically significant.

Results

One hundred and sixty-eight lung adenocarcinoma resections were included in the study; these included four wedge

resections, four with multiple wedge resections, four segmentectomies, two partial lobectomies, 144 lobectomies, and ten pneumonectomies. The number of H&E-stained slides from lung parenchyma uninvolved by and at a distance greater than 1 cm from the pulmonary malignancy that were examined varied from two to ten per case. On histologic review, 65 cases (38.7%) showed SRIF in the lung uninvolved by tumor; 103 (61.3%) cases did not show SRIF. In cases with SRIF, alveolar septal fibrosis with dense, eosinophilic, paucicellular collagen bundles was present in the subpleural lung parenchyma with centrilobular peribronchiolar accentuation (Figure 1). The findings of SRIF were distributed in all lung lobes (Table 1). Among cases with SRIF, 61 (93.8%) had concomitant emphysema on histologic examination. In these cases, SRIF was seen both in and away from the emphysematous lung parenchyma. In contrast, 85 (82.5%) patients without SRIF had emphysema on histologic exam (P=0.04). Of note, all cases showed patchy evidence of respiratory bronchiolitis.

A history of cigarette smoking was documented in all cases. The smoking history elicited from patients with and without SRIF was 39.8±19.8 pack years and 37.5±22.5 pack years, respectively (P=0.41) (Table 1). The average age at resection among patients with and without SRIF was 66±8 years and 67±10 years, respectively (P=0.85). There was no significant difference in the sex of patients with or without SRIF (70.8% vs 62.1%, respectively; P=0.32). Results of imaging studies were not consistently available for review; however, few patients had evidence of SRIF on computed tomography (CT) evaluation (Figure 2).

Genetic analysis

One of three sequentially updated versions of SNaPshot and direct PCR sequencing assays were performed on 64 cases with SRIF and 102 cases without SRIF. *ALK* FISH, *ROS* FISH, and *MET* FISH were performed on 64, 16, and 13 cases with SRIF, respectively, and on 99, 20, and 18 cases without SRIF, respectively. There was no difference in the number of mutations present in each of the 14 interrogated genes or in the prevalence of individual hotspot mutations in cases with and without SRIF. Mutations in *EGFR* were observed in 9 (14.1%) cases with SRIF and 19 (18.6%) cases without SRIF (P=0.53); mutations in *KRAS* occurred in 24 (37.5%) cases with SRIF and in 50 (49.0%) cases without SRIF (P=0.15) (Table 1). Finally, there was no difference in the number of cases with and without SRIF in which no genetic mutation was identified (27 and 30, respectively; P=0.13).

Discussion

SRIF was present in the background lung parenchyma of 38.7% of lungs from smokers with pulmonary adenocarcinoma. This is comparable to other recently reported studies of SRIF.[12–15] Katzenstein et al evaluated lobectomy specimens resected for a malignant neoplasm in 20 smokers and identified patchy widening of alveolar septa by acellular collagen deposition with minimal or focal associated inflammation in 45% of cases.[12] SRIF was predominantly subpleural, but also occurred with or without associated emphysema in deep lung parenchyma in a centrilobular distribution, as in the present study. Similar lesions had previously been described in up to 50% of smokers, although authors have ascribed different terms to comparable histologic findings, including "respiratory bronchiolitis-associated interstitial lung disease with fibrosis" and "airspace enlargement with fibrosis".[13–15] These interstitial changes were absent in nonsmokers and must be distinguished from other fibrosing interstitial lung diseases, including IPF and nonspecific interstitial pneumonia,

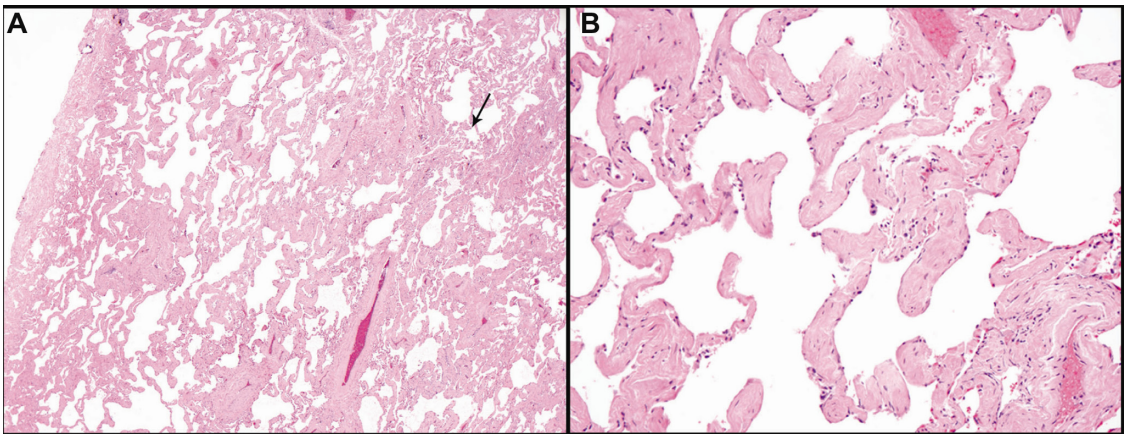

Figure 1 (**A**) Smoking-related interstitial fibrosis involving subpleural lung parenchyma (pleural surface is at the left) with centrilobular accentuation (arrow); (**B**) thickening of the alveolar septa by dense, eosinophilic, paucicellular collagen bundles.

Table 1 Clinical and pathologic features of pulmonary adenocarcinoma cases with and without SRIF

Features	With SRIF (n=65)	Without SRIF (n=103)	*P*-value
Female	46 (70.8%)	64 (62.1%)	NS
Male	19 (29.2%)	39 (37.9%)	NS
Average age (years)	66±8	67±10	NS
Smoking history (pack-years)	39.8±19.8	37.5±22.5	NS
Emphysema	61 (93.8%)	85 (82.5%)	0.04
Distribution of examined uninvolved lung parenchyma			
Right upper lobe	23 (35.4%)	33 (32.0%)	NS
Right middle lobe	6 (9.2%)	5 (4.9%)	NS
Right lower lobe	10 (15.4%)	19 (18.4%)	NS
Left upper lobe	13 (20.0%)	22 (21.4%)	NS
Left lower lobe	6 (9.2%)	14 (13.6%)	NS
Unspecified lobe	7 (10.8%)	10 (9.7%)	NS
EGFR mutations*	9 (14.1%)	19 (18.6%)	NS
KRAS mutations*	24 (37.5%)	50 (49.0%)	NS

Note: *Of 64 cases with SRIF and 102 cases without SRIF.
Abbreviations: NS, not significant; SRIF, smoking-related interstitial fibrosis.

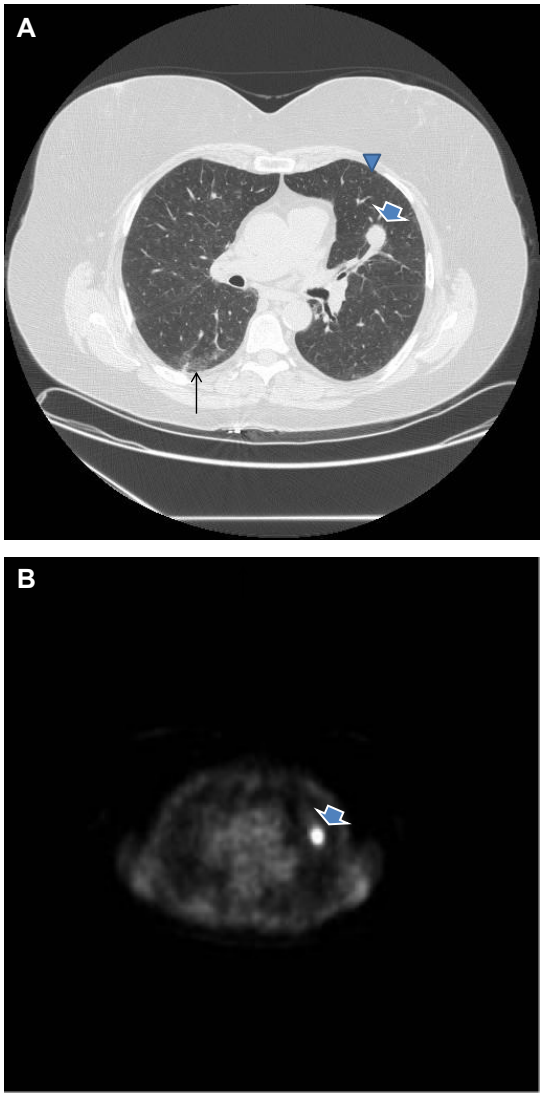

Figure 2 Spiculated nodule in the left upper lobe (arrow in **A**) with associated 18-FDG uptake on positron emission tomography scan (**B**), consistent with lung cancer.
Notes: Note subtle centrilobular nodules (arrowhead) and subpleural reticulation and ground glass opacity (arrow) on the computed tomography scan (**A**), consistent with smoking-related interstitial fibrosis.

which have other important clinical implications.[4,5,13–15] The distinction of SRIF from other chronic interstitial fibrosing lesions not only has prognostic implications but also affects therapeutic interventions.

While SRIF seems to be a common finding in smokers, clinical symptoms and radiologic findings associated with this pattern of interstitial lung disease are uncommon.[12–14] However, the index group of patients with SRIF described by Yousem et al presented with signs and symptoms of chronic interstitial lung disease and, for this reason, underwent lung biopsy.[13] Katzenstein et al identified mild to moderate reduction in diffusion capacity in four of nine (44%) patients with SRIF who underwent pulmonary function testing.[12] Notably, several radiology studies have reviewed CT findings in smokers and found subtle interstitial changes in a subset of smokers. Lederer et al found ground glass and reticular opacities on CT scan in 2.2% of smokers, with increasing numbers of these areas in patients with increasing pack years.[16] More recently, Washko et al and Sverzellati et al reported interstitial lung abnormalities in 8% and 22.8% of smokers, respectively.[17,18] Thus, while SRIF is often an incidental finding recognized in lung parenchyma, these studies suggest that the increasing use of high-resolution CT may increase the detection of interstitial lung abnormalities in asymptomatic smokers and lung biopsies may be utilized to provide insight into the etiology of a patient's pulmonary interstitial abnormalities.

It is possible that SRIF is part of the spectrum of changes seen in emphysema as, in this study, emphysema was more often present in cases with SRIF than in those without SRIF. Smokers with both pulmonary fibrosis and emphysema have been previously described.[7,19–22] Cottin et al coined the term "combined pulmonary fibrosis and emphysema" (CPFE) in a retrospective study of 61 patients with upper lobe emphysema and lower lobe pulmonary fibrosis on high-resolution CT scan.[7] Notably, CPFE has been associated with an increased risk of lung cancer. Kitaguchi et al reported a 46.8% prevalence of lung cancer in patients with CPFE as compared to 7.3% in patients with chronic obstructive pulmonary disease alone.[8] More recently, Usui et al evaluated 1,143 consecutive patients with primary lung carcinoma and found that 8.9% had evidence of CPFE

on CT scan at the time of cancer diagnosis.[9] This association between CPFE and lung cancer may reflect the importance of cigarette smoking as a predisposing factor. However, the presence of interstitial fibrosis might be an independent factor in the development and progression of lung cancer.[9,10]

In 1939, Friedrich[23] first proposed the association between pulmonary fibrosis and lung cancer. Subsequent studies have supported this relationship.[2–4] IPF has been implicated as an independent risk factor for the development of lung cancer, although there is an increased incidence of IPF in smokers.[1,24–30] Furthermore, molecular studies have demonstrated the presence of genetic alterations, especially in tumor suppressor genes such as *p53*, within lung tissue from patients with IPF.[31–36] Thus, it is hypothesized that repeated inflammation, injury, and repair of the lung parenchyma may predispose to the development of lung cancer. More recently, inappropriate cytokine signaling has been implicated as a causative mechanism of IPF and may play a role in pulmonary carcinogenesis.[37]

Lung cancer in the setting of ILD is the most common malignancy to develop in patients with systemic sclerosis.[38–42] Similarly to IPF, lung cancer in these patients may result from cellular injury and genetic damage caused by continued pulmonary inflammation and fibrosis. In addition, other rheumatologic diseases, such as dermatomyositis, rheumatoid arthritis, systemic lupus erythematosus, and sarcoidosis, and pneumoconioses, including asbestosis, silicosis, and berylliosis, have been associated with an increased risk of the development of lung cancer.[2–4] Notably, although the literature is controversial, studies have suggested that, among patients with asbestosis, there is an increased prevalence of tumors within the lower lung lobes; ie, in the most fibrotic regions of the lung parenchyma.[43,44]

In light of the strong association of pulmonary fibrosis and lung cancer, we hypothesized that SRIF might be associated with a distinct molecular pathway in the development of lung cancer in smokers, despite the underlying relationship between both SRIF and cancer with cigarette smoking. Recent studies have showed an inverse relationship between the presence of interstitial lung disease and *EGFR* mutations in associated lung tumors.[45,46] However, unlike our study, these studies did not specifically evaluate the role of SRIF in cancer progression, as both smokers and nonsmokers and patients with multiple subtypes of interstitial fibrosis were included in these studies. We found no difference in the number or types of genetic mutations in adenocarcinomas that develop in lungs with and without SRIF, which suggests that there is no difference in the molecular pathogenesis of adenocarcinomas that arise within smokers in the presence of SRIF. However, due to the limited sample size, additional research must be done to further investigate the potential clinical significance of SRIF and subsequent impact on cancer progression. In addition, the mechanism by which other fibrosing lung diseases, such as IPF, can lead to cancer development must be clarified.

This study has several limitations. Firstly, information regarding results of pulmonary function tests and imaging studies were not consistently available for review. A comparison of the mutations present in tumors from patients in our cohort with normal and abnormal lung function and those with and without evidence of interstitial fibrosis on imaging may have proved insightful. Secondly, this study was retrospective and nonrandomized. Most mutations, such as rearrangements in *ALK* and *ROS*, occurred in only small subsets of lung adenocarcinomas, so that it was not possible to make statistically relevant conclusions concerning these cases. A prospective randomized controlled study with systematic sampling of both subpleural and deep lung parenchyma away from the tumor would allow for more accurate assessment of the presence of SRIF. In addition, limited numbers of genes were included in the SNaPshot and FISH analysis that were performed, such that other mutations of possible relevance were not interrogated. Future studies adopting broad next-generation sequencing techniques may yield greater insight into the possible role of SRIF in the pathways of carcinogenesis. It is, of course, possible that the same carcinogens that lead to mutations in the lung epithelial genome are responsible for SRIF, but this remains to be investigated. However, despite the limitations of the present study, it is, to our knowledge, the first to explore the possible relationship between SRIF and the pathogenesis of lung cancer.

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Disclosure

DDS and AJI serve as consultants for Bioreference Laboratories. The authors declare no further conflicts of interest in this work.

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